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Characterization of Hepatitis B Virus Mutations in Untreated Patients Co-Infected With HIV and HBV Based on Complete Genome Sequencing

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Co-infection of HBV with HIV results in an accelerated course of HBV-associated chronic liver disease. Several studies have shown that viral mutations are related to disease progression in mono-infection with HBV. However, it is unclear whether HBV mutation patterns might differ between co-infected and mono-infected patients. To compare the frequencies and mutation patterns in the HBV genome between co-infection and mono-infection. Twenty-four treatment-naïve co-infected and 31 treatment-naïve mono-infected Thai patients were included. HBV mutations were characterized by whole genome sequencing of virus serum samples. The clinical features and frequency of known clinically significant mutations were compared between the two groups. No significant difference between the groups was found with respect to sex, age and HBeAg. However, HBV DNA levels were significantly higher in co-infected patients. The distribution of HBV genotypes was comparable between the two groups and restricted mostly to sub-genotypes C1 and B2. An isolate with recombinants of genotypes G/C1 was also identified in a patient with co-infection. There was no difference in the prevalence of mutations in the enhancer II/basal core promoter/precore region, *pre-S/S* and polymerase genes between the two groups. In conclusion, dual infections tend to engender increased HBV DNA levels. There was no major difference in the frequencies of common HBV mutations between co-infected and mono-infected patients. Thus, HBV mutations may not contribute to disease pathogenesis in Thai patients with co-infection. ***J. Med. Virol. 85:16–25, 2013.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: hepatitis B virus; HIV; mutations; genotypes; genome sequencing; recombination

INTRODUCTION

Co-infection with HIV and HBV is common because both viruses share routes of transmission through blood or blood products and sexual contact. There are approximately 40 and 400 million people worldwide infected chronically with HIV and HBV, respectively [McGovern, 2007; Thio, 2009]. An estimated 10% of HIV-infected individuals have chronic hepatitis B, with a higher prevalence found in areas where HBV infection is endemic including countries in Africa and

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Ethical approval: The study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

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Asia. Following the introduction of HAART, liver disease has emerged as one of the most important causes of morbidity and mortality in HIV–HBV co-infected populations [Thio, 2009; Joshi et al., 2011]. It is well recognized that HIV infection can accelerate the course of HBV-associated liver disease, such as the progression of cirrhosis in co-infected individuals [Price and Thio, 2010]. In contrast, the association between HIV infection and the occurrence of HBV mutations is less well known.

HBV shows remarkable genetic variability and is classified currently into 10 genotypes, designated A–J based on genome sequence analysis [Liaw and Chu, 2009]. The virus has a high mutation rate compared with other DNA viruses as it lacks proofreading capacity during its replication via reverse transcription [Kay and Zoulim, 2007]. Several studies have shown that mutation patterns in the viral genome are associated significantly with progression of liver disease in HBV mono-infection. The most well known occurring naturally mutations include PC stop codon mutation (G1896A), which abolishes HBeAg production, and double BCP mutations (A1762T/G1764A), which down regulate HBeAg production. Another PC mutation is G1899A, which often occurs either alone or with the G1896A mutation. Recent data in HBV mono-infection have shown that the double BCP mutations are associated with more aggressive liver disease and the development of HCC [Wai and Fontana, 2004; Lin and Kao, 2008]. Other mutations such as T1753C/A/G in the BCP region and C1653T in the EnhII region have also been recognized as being associated with the outcome of chronic HBV mono-infection [Tanaka et al., 2006; Yuen et al., 2008]. Furthermore, mutations or deletions in the *pre-S1*/*pre-S2* gene have been shown to accumulate frequently in HBV mono-infected patients and are associated with an increased risk of HCC [Chen et al., 2007; Choi et al., 2007]. Whether these variants might be more found frequently in HIV–HBV co-infection has been investigated poorly. The aim of this study was to identify mutation patterns by complete genome sequencing of HBV among Thai patients co-infected with HIV compared to sex- and age-matched mono infected individuals.

MATERIALS AND METHODS

Study Patients

The co-infected patients enrolled in this study were part of a prospective observational study of all HIV-infected patients who participated in any clinical/observational trial of the HIV Netherlands Australia Thailand Research Collaboration (HIV-NAT), Bangkok, Thailand from April 1999 to October 2008. All 24 individuals recruited for this study were chosen on the basis of serum availability and detectable HBV DNA level. To compare HBV genome sequences between co-infected and mono-infected individuals, serum samples were also obtained from 31 patients with chronic HBV mono-infection, who were matched for age (±5 years) and gender. These patients were selected randomly from a pool of patients with chronic liver disease who were followed-up at King Chulalongkorn Memorial Hospital (Bangkok, Thailand) during the same period.

None of the patients enrolled in this study had hepatitis C virus infection or received any antiviral therapy for chronic HBV infection when the serum sample was obtained. All patients were informed about the purpose of the study, and subsequently gave their written informed consent. Serum samples were collected and frozen at −70°C until further use. The study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

HBV Genome Sequencing

HBV DNA was extracted from 100-μl plasma samples using proteinase K in lysis buffer followed by phenol/chloroform extraction and ethanol precipitation. The DNA pellets were dissolved in 30 μl sterile distilled water. Subsequently, serum samples were subjected to complete HBV genome amplification by PCR using four primer sets selected from conserved regions as previously described [Sa-Nguanmoo et al., 2008]. The primer sequences were as follows:

Set 1, PreS1F+ (5′-GGGTCACCATATTCTTGGGAAC-3′: position 2814 to 2835) and R5 (5′-AGCCCAAAA-GACCCACAATTC-3′: position 1015 to 995);
Set 2, F6 (5′-ATATGGATGATGTGGTATTGGG-3′: position 737 to 758) and X102 (5′-ACCTTTAACC-TAATCTCC-3′: position 1764 to 1748);
Set 3, X101 (5′-TCTGTGCCTTCTCATCTG-3′: position 1552 to 1569) and CORE2 (5′-CCCACCTTAT-GAGTCCAAGG-3′: position 2476 to 2457);
Set 4, CORE1 (5′-GAGTGTGGATTCGCACTCCTCC-3′: position 2268 to 2289) and R1 (5′-TGTAACAC-GAGCAGGGGTCCTA-3′: position 201 to 180).

The PCR reaction mixture and the amplification method were performed as described previously [Sa-Nguanmoo et al., 2012]. The sequencing was performed by a commercial sequencing assay (First BASE Laboratories Sdn Bhd, Selangor Darul Ehsan, Malaysia). Nucleotide sequences were edited and assembled using SEQMAN (LASERGENE program package, DNASTAR) and submitted to the GenBank database (accession no. JQ801470-JQ801524).

Genotyping, Phylogenetic Analysis, and Mutation Analysis

The sequences were characterized for the genotyping assay by phylogenetic analysis as previously described [Sa-Nguanmoo et al., 2010]. Briefly, each sample's sequences from *pre-S1*/*pre-S2*/*S* and *preC*/

C regions were aligned with each available human genotype stored at the GenBank database (National Center for Biotechnology Information, Bethesda, MD) by Clustal X program version 2.0.10 (European Bioinformatics Institute, Cambridge, UK). Based on these alignments, phylogenetic trees were constructed for genotyping using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (The Biodesign Institute, Tempe, AZ). Some sequences were genotyped by the Viral Genotyping Tool (National Center for Biotechnology Information). Genetic recombinants were further determined by SimPlot program and bootscanning analysis (Simplot Version 3.5.1, Baltimore, MD). The nucleotides were translated into amino acid sequences using the translation tool in ExPASy Proteomics Server (http://www.expasy.ch/tools/dna.html).

Complete genome sequences were evaluated for mutations and deletions in the EnhII/BCP/PC region, *pre-S*/*S* and polymerase genes. The amino acids at positions 120 and 160 of the S protein were indicative for "a" determinant mutations.

HBV DNA Quantitation

Serum HBV DNA level was measured by a commercial real-time method with a detection limit as low as 10 IU/ml (Roche Diagnostics, Tokyo, Japan) in accordance with the manufacturer's instructions.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD), and percentages as appropriate. Comparisons between groups were analyzed by the χ^2 or Fisher's exact test for categorical variables and by the Mann–Whitney *U*-test or Student's *t*-test for quantitative variables. *P*-values < 0.05 were considered statistically significant. Data were analyzed by using the SPSS software for Windows 17.0 (SPSS, Inc., Chicago, IL).

RESULTS

Demographic and Clinical Data of the Patients

The demographic data and clinical features of patients with co-infection and mono-infection are shown in Table I. Compared with the mono-infection group, patients with co-infection had higher significantly mean levels of HBV DNA. However, there was no significant difference between groups with respect to sex, age, serum ALT level and status of HBeAg (positive/negative).

Distribution of HBV Genotypes

Phylogenetic analysis was performed based on the *pre-S1*/*pre-S2*/*S* and *preC*/*C* genes (Fig. 1A,B). Among co-infected patients, 18 (75%) samples were determined as genotype C (sub-genotype C1) and 5 (20.8%) samples were identified as genotype B (sub-genotype B2). As for mono-infected patients, 24 (77.4%) and 7 (22.6%) samples were identified as genotype C (sub-genotype C1) and genotype B (sub-genotype B2), respectively. There was no statistical difference in the distribution of HBV genotypes between the two groups (Table II).

Based on the whole genome sequence, recombination of genotypes G and C1 was identified in a patient with co-infection. The recombination breakpoints were estimated at positions 46 (*pre-S2*/*pol* overlapping region), 760 (*S*/*pol* overlapping region), 1540 (*X*/*pol* overlapping region), and 2780 (*pol* gene; Fig. 2).

Prevalence and Characterization of EnhII/BCP/PC Mutations

Common mutations were found at nucleotides 1653, 1753, 1762, 1764, 1858, 1896, and 1899. The C1653T mutation/deletion was observed in 2 (8.3%) samples of co-infected patients but was not detected in mono-infected patients. The T1753C mutation/deletion was observed in 5 (20.8%) and 5 (16.1%) samples obtained from co-infected and mono-infected patients,

TABLE I. Demographic and Clinical Characteristics of Co-Infected and Mono-Infected Patients

Characteristics	Co-infection (n = 24)	Mono-infection (n = 31)	*P*
Age, years	36.4 ± 8.3	37.8 ± 7.7	0.517
Sex			
Male	18 (75)	23 (74.2)	0.598
Female	6 (25)	8 (25.8)	
ALT, U/L	100.4 ± 134.2	96.7 ± 61.1	0.902
HBeAg status			
Positive	13 (54.2)	16 (51.6)	0.534
Negative	11 (45.8)	15 (48.4)	
HBV DNA level, $\log_{10}$ IU/ml	7.0 ± 1.9	6.0 ± 1.5	0.039
CD4 count, cell/μl	243.0 ± 195.5		
HIV RNA level, log10 copies/ml	4.2 ± 1.3		
Risk factors for HIV infection			
Heterosexual	18 (75)		
Homosexual	6 (25)		

Data were expressed as mean ± SD, no. (%).

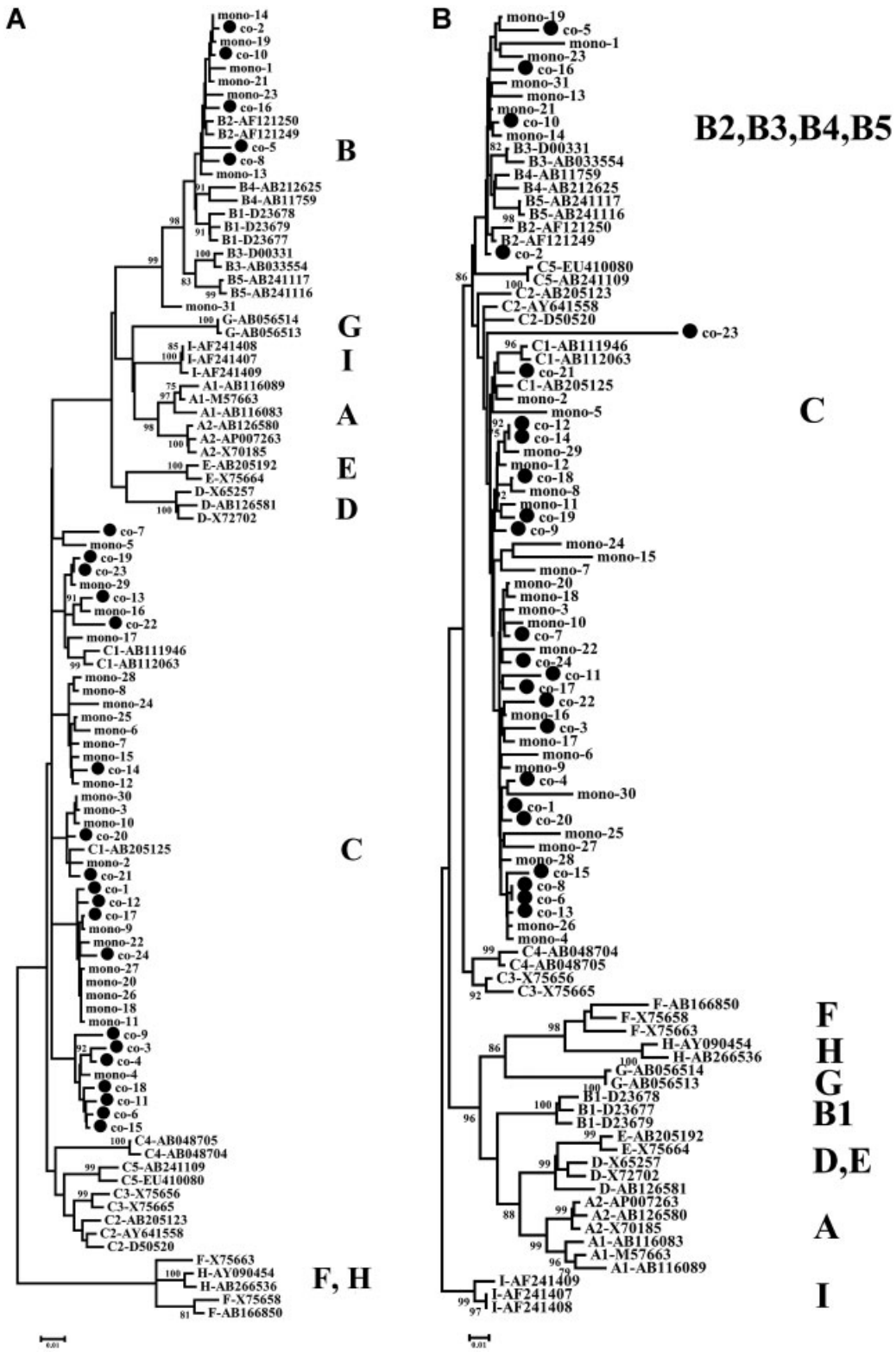

Fig. 1. Phylogenetic relationship of the sequence obtained in the present study and representative sequences of human HBV strains from GenBank. Regions included in the comparison were: (**A**) the *pre-S1*/*pre-S2*/*S* gene; (**B**) the *preC*/*C* gene, including nucleotide positions 1814–2452. Percentage bootstrap values (>75%) are shown at the respective nodes. The scale bar at the bottom indicates the genetic distance. The sequences in this study are indicated by symbols (•co-xx, co-infection; mono-xx, mono-infection**).**

TABLE II. Prevalence of HBV Mutations in Co-Infected and Mono-Infected Patients

Characteristics	Co-infection (n = 24)	Mono-infection (n = 31)	*P*
HBV genotype			0.517
B2	5 (20.8)	7 (22.6)	
C1	18 (75)	24 (77.4)	
G/C1	1 (4.2)	0	
C1653T/deletion	2 (8.3)	0	0.102
T1753C/deletion	5 (20.8)	5 (16.1)	0.654
A1762T/deletion	11 (45.8)	13 (41.9)	0.773
G1764A/deletion	12 (50)	13 (41.9)	0.551
T1858C/deletion	1 (4.2)	1 (3.2)	0.853
G1896A/deletion	6 (25)	11(35.5)	0.404
G1899A/deletion	1 (4.2)	2 (6.5)	0.711
Pre-S1 mutation/deletion	4 (16.7)	4 (12.9)	0.695
Pre-S2 mutation/deletion	5 (20.8)	6 (19.4)	0.892
"a" determinant mutation	1 (4.2)	0	0.251
Drug resistance mutation			0.790
rtI233V	1 (4.2)	1 (3.2)	
rtV173L	0	1 (3.2)	

Data were expressed as no. (%).

respectively. The A1762T and G1764A mutations were found in 11 (45.8%) and 12 (50%) samples of co-infected patients, while the double mutations were found in 13 (41.9%) samples of mono-infected patients. The T1858C mutation was found in 1 (4.2%) and 1 (3.2%) sample of co-infected and mono-infected patients, respectively. The G1896A mutation was found in 6 (24.6%) and 11 (35.5%) samples of co-infected and mono-infected patients, respectively. In addition, The G1899A mutation was detected in 1 (4.2%) sample of co-infected patients and 2 (6.5%) samples of mono-infected patients. There was no significant difference in the prevalence of these mutations between the two groups (Table II). Nucleotide sequence alignment of the EnhII/BCP/PC region of these 55 samples is shown in Figure 3.

Prevalence and Characterization of *pre-S*/*S* Mutations/Deletions

Pre-S1 mutations/deletions were detected in 4 (16.7%) and 4 (12.9%) samples of co-infected and

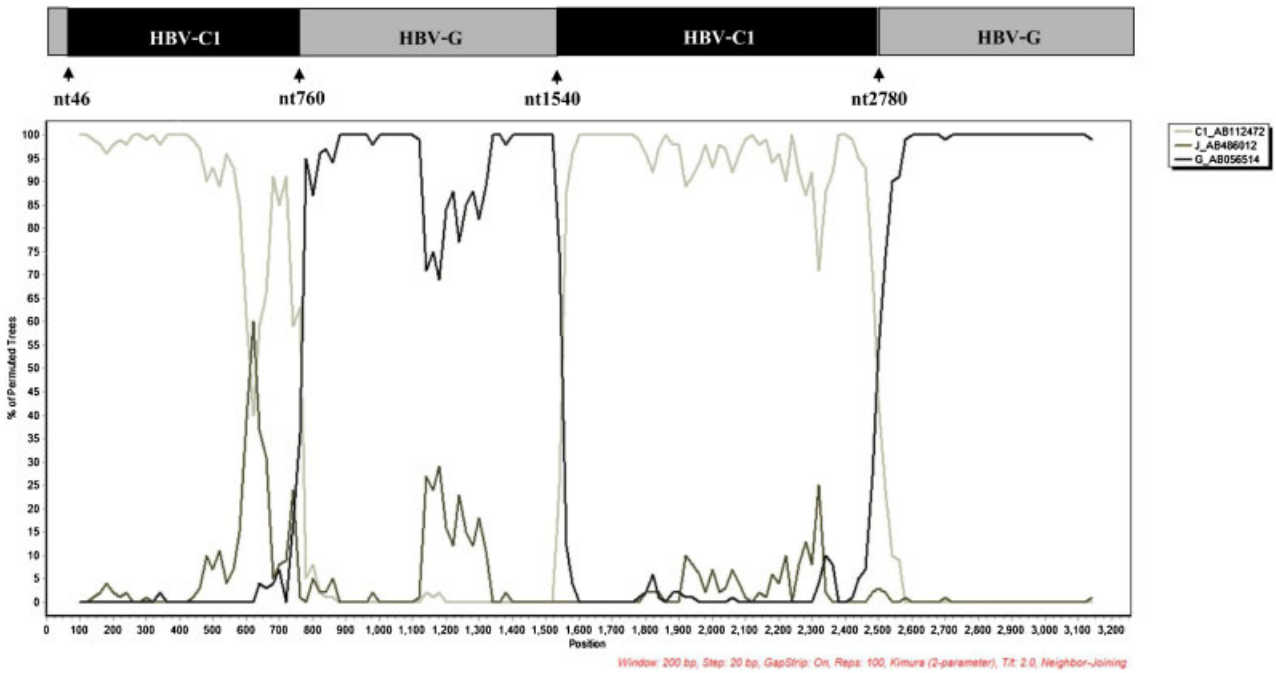

Fig. 2. Bootscanning analysis of the recombinant isolate. Dashed line(s) indicate(s) the breaking point(s) of recombination. The number above the dashed line indicates the nucleotide position of each isolate compared with the reference strain (accession no. C1-AB112472, G-AB056514) and the outgroup strain (accession no. J-AB486012).

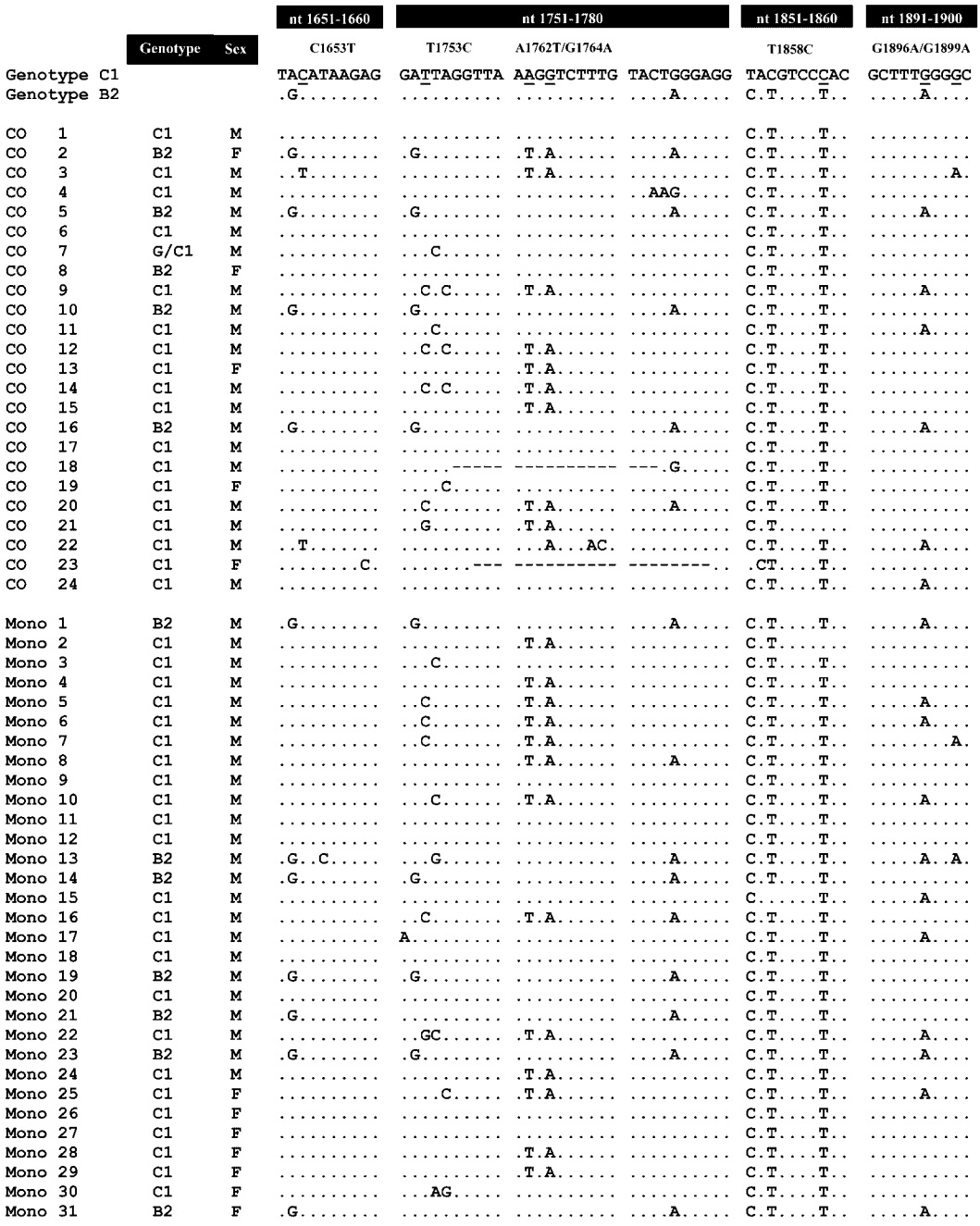

Fig. 3. Nucleotide sequence alignment of the EnhII/BCP/PC region of the samples in this study.

mono-infected patients, respectively. The prevalence of *pre-S2* mutations/deletions among co-infected and mono-infected patients was 20.8% and 19.4%, respectively. There was no significant difference in the prevalence of such mutations between the two groups (Table II). As for the prevalence of site-specific *pre-S*/*S* mutations, *pre-S1* deletion and *pre-S2* start codon mutation was the most common (18.2% each), followed by *pre-S2* deletion (14.5%) and *pre-S1* start codon mutation (10.9%). The mean age of patients with *pre-S* mutations/deletions was higher significantly than that of patients without the mutants (39.5 ± 9.7 years vs. 31.4 ± 11.2 years, $P = 0.035$). Amino acid sequence alignment of the pre-S1/pre-S2 region of these 55 samples is shown in Figure 4.

Prevalence and Characterization of the "a" Determinant and Drug Resistance Mutations

A point mutation (Gly145Arg) in the "a" determinant region was detected in one (4.2%) sample

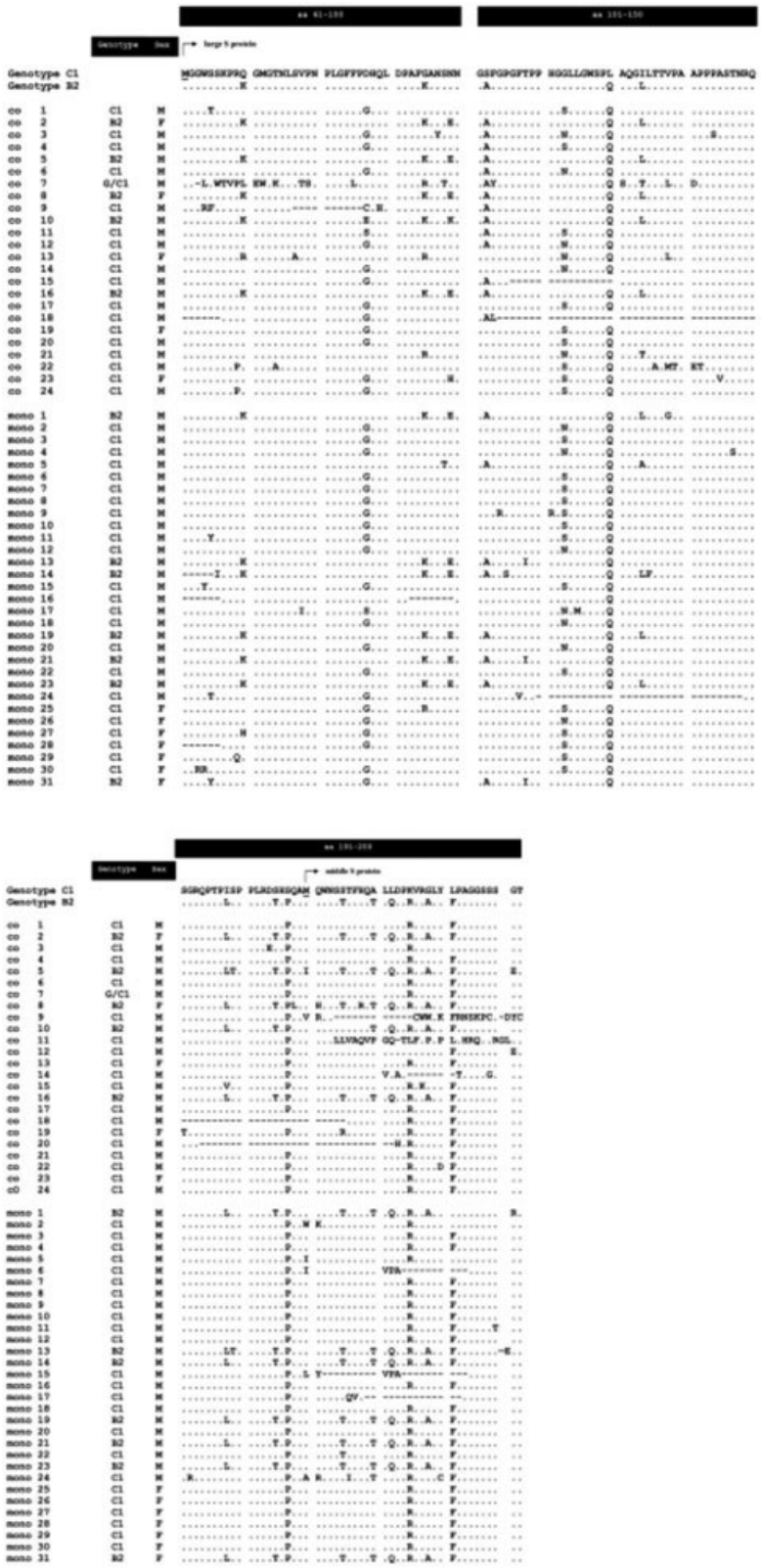

Fig. 4. Amino acid sequence alignment of the pre-S1/pre-S2 region of the samples in this study.

obtained from co-infected patients. As for mutations in the polymerase gene, a point mutation associated with adefovir resistance (rtI233V) was found at similar frequency among co-infected and mono-infected patients (4.2% and 3.2%, respectively). Moreover, a mutant associated with lamivudine resistance (rtV173L) was detected in one (3.2%) of mono-infected patients but was not found in co-infected patients (Table II).

Comparison of Mutations in Relation to HBV Genotypes

To determine whether the differences in frequencies of EnhII/BCP/PC and *pre-S* mutations/deletions between the co-infected and mono-infected patients were influenced by HBV genotypes, the mutations within each group were compared with respect to genotypes B and C. There was no significant difference in the prevalence of these mutations between the two groups depending on HBV genotype (Table III).

DISCUSSION

Although HIV–HBV co-infection prevails in Southeast Asia, it is unclear whether the complete HBV genome may differ between co-infected and mono-infected individuals. Previous data have demonstrated that an HBV mutant encoding truncated core/precore proteins (−1G mutant) was associated with high HBV DNA levels in co-infected patients with genotype A [Revill et al., 2007]. A recent study from several sites including Australia, the United States, and Asian countries showed that HBV mutations varied significantly between co-infected and mono-infected patients when compared with the same HBV genotype. In particular, double BCP mutations were more frequent significantly in HBV genotype C mono- infection and the −1G mutant was more frequent significantly in co-infection and was observed only in genotype A. Furthermore, *pre-S2* deletions were detected more frequently in co-infected samples [Audsley et al., 2010]. A previous prospective study from Argentina showed that isolates of HBV genotypes A and F from mono-infected patients tended to have BCP mutations comparable to those of co-infected patients [Cassino et al., 2009]. These data suggest HBV mutation patterns might be associated with HIV co-infection and might be contributing to disease pathogenesis in co-infected individuals.

The current study was designed to exclude the possibility of cohort effect by sex- and age-matching between Thai patients with co-infection and mono-infection. In addition, confounding factors of HBV genotypes and HBeAg status were minimized as they were also matched between the two groups. This data confirmed previous reports that HBV viral load is higher typically in co-infection than mono-infection [Colin et al., 1999]. In contrast, this study showed that there was no significant difference in the frequencies of double BCP mutations between the two groups although such mutations were more found commonly in genotype C. In addition, the prevalence of G1896A, T1753C, and C1653T was not significantly different between the two groups. It should be mentioned that HBV genotypes in this study were restricted mostly to sub-genotypes C1 and B2, which reflected their high frequencies in Southeast Asia and Thailand [Tangkijvanich et al., 2005; Sa-Nguanmoo et al., 2010]. As a result, the occurrence of −1G mutant, which is restricted to genotype A, could not be detected in this study.

It has been shown that *pre-S* deletion mutants tend to accumulate during a later stage of persistent HBV infection, including cirrhosis and HCC [Chen et al., 2006; Choi et al., 2007]. Data from in vitro studies have demonstrated that intracellular accumulation of pre-S mutant proteins can induce ER stress and alter HBV protein expression, which are associated with increased oxidative stress and DNA damage [Hsieh et al., 2004]. This study showed rather similar prevalence of these mutants in both groups, although they were more found commonly in genotype C. Moreover, patients with *pre-S* mutations/deletions were older than those infected with the wild-type, which confirmed previous observations that the frequency of the mutants tends to increase in direct relation to the patient's age [Chen et al., 2006; Choi et al., 2007]. Taken together, this study suggests that the emergence of common mutations in the EnhII/BCP/PC and *pre-S* regions might not lead to pathogenesis in

TABLE III. Prevalence of HBV Mutations According to HBV Genotypes

	Co-infection (n = 24)		Mono-infection (n = 31)	
Characteristics	B2 (n = 5)	C1 (n = 19)[a]	B2 (n = 7)	C1 (n = 24)
C1653T	0	2 (10.5)	0	0
T1753C	0	5 (26.3)	0	5 (20.8)
A1762T	2 (40)	9 (47.4)	0	13 (54.2)
G1764A	2 (40)	10 (52.6)	0	13 (54.2)
T1858C/deletion	0	1 (5.3)	0	1 (4.2)
G1896A	2 (40)	4 (21.1)	3 (42.9)	8 (33.3)
G1899A/deletion	0	1 (5.3)	1 (14.3)	1 (4.2)
Pre-S mutations/deletions	0	7 (36.8)	1 (14.3)	8 (33.3)

Data were expressed as no. (%).
[a]Included one case of genotype G/C1 recombination.

co-infected patients. Instead, these mutants might occur during a long-standing inflammatory process of chronic HBV infection among Thai populations.

Amino acid substitutions within the "a" determinant domain lead to conformational changes which may interfere with immunization against HBV infection [Coleman, 2006]. Indeed, the G145A mutant in the second loop of the "a" determinant is considered the most common mutation causing vaccine escape. The prevalence of the G145A mutant among co-infected patients in this study was 4% approximately, which was lower slightly than that reported previously in mono-infected individuals (6–12%) [Coleman, 2006]. Regarding polymerase gene mutations, several signature mutants associated with drug resistance have been characterized. Such mutations are classified as primary (being responsible for decreased drug susceptibility) or compensatory (being responsible for restoring replication fitness of the mutants) [Ghany and Liang, 2007]. In this study, a primary mutation associated with adefovir resistance (rtI233V) was found at similar frequency in the two groups. However, the primary mutation associated with lamivudine resistance was not detected; instead, a compensatory mutant (rtV173L) was found in a patients with mono-infection. This finding was in contrast to most reports but rather similar to a recent study conducted in co-infected patients [Tuma et al., 2011].

HBV strains resulting from genome recombination between different genotypes have been recognized increasingly [Simmonds and Midgley, 2005]. In this study, recombination between genotypes G and C1 was identified in a co-infected patient based on its unusual genotyping pattern upon sequencing and phylogenetic analysis. Indeed, recombination between genotypes C and G appears unusual in Asia as these genotypes are subject to distinct geographical distributions. Interestingly, the site of breakpoints of this recombinant differed from that found in a hybrid of genotypes G/C previously described [Suwannakarn et al., 2005]. It has been proposed that HBV genotype G requires dual infections with other strains to sustain chronicity, although replication competence of this genotype has been documented [Li et al., 2007]. A recent report from the United States has shown that non-hybrid HBV genotype G infection was found frequently in co-infected patients and was associated with more rapid disease progression [Dao et al., 2011]. Whether the recombinant strain of genotypes G/C does enhance the pathogenic potential of the viruses through inter-genotype recombination is unclear and requires further investigation.

In conclusion, this study has shown that dual infections tend to engender increased HBV DNA levels. There was no major difference in the frequencies of common HBV mutations between co-infected and mono-infected patients and these mutants might not be contributing to disease pathogenesis in co-infected patients. However, further large-scale prospective studies, which offer advantages over cross-sectional investigations, are required to confirm these observations.

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